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Effects of Valine on Protein Synthesis and Turnover in *Pseudomonas saccharophila* under "Nongratuitous" Inducing Conditions

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Under "nongratuitous" inducing conditions, in *Pseudomonas saccharophila*, D- and L-valine and L-isoleucine inhibit net protein synthesis. At a concentration of 0.5 μmole or greater of valine per mg of bacterial protein, net protein synthesis declined approximately 70%. The inhibitory effect of valine is proportional to the exogenous valine concentration. Studies of ^{14}C amino acid incorporation and ^{14}C amino acid release from prelabeled cells indicate that valine stimulates protein turnover.

An inhibitory effect of valine on the growth of *Bacillus anthracis* was reported by Gladstone in 1939 (5). Similarly, leucine and isoleucine added singly to a synthetic medium inhibit the growth of this microorganism. An inhibitory effect of L-valine on the growth of *Escherichia coli* K-12 was also reported (2). Umbarger and Brown (15) suggested that the mechanism of inhibition is the interference of excess valine with the biosynthesis of isoleucine. Repression by valine, acting in concert with isoleucine and leucine, of the enzymes involved in the biosynthesis of valine and isoleucine has also been reported for different microorganisms (1, 4).

Recently, Temple et al. (14) showed that L-valine inhibits the overall rate of protein synthesis and also the synthesis of β-galactosidase during induction in the presence of glycerol in *E. coli* K-12. In contrast, *Pseudomonas saccharophila* is only slightly inhibited by valine during ordinary growth. After a shift to a new carbon source, however, the organism becomes sensitive to high valine concentrations. The work on *P. saccharophila* presented here reveals that, under these conditions, protein turnover is stimulated.

MATERIALS AND METHODS

Organism. Amino acids were obtained from Calbiochem, Los Angeles, Calif., and the radioactive amino acids were purchased from Volk Radiochemical Co., Los Angeles, Calif.

Stock cultures of *P. saccharophila* were maintained in the growth medium described previously, with 0.2% sucrose as the carbon source (11).

Experimental culture. To prepare inocula for experimental cultures, 5 ml of stock culture was added to 45 ml of growth medium containing 0.2% sodium lactate instead of 0.2% sucrose as the carbon source, and was shaken for 24 hr at 30 C. The resulting culture was then used as the inoculum for 500 ml of growth medium containing 0.2% lactate. After 18 hr, the bacterial cells were sedimented at 4,000 × *g* for 10 min and washed in a volume of 0.033 M phosphate buffer (*p*H 6.8) equal to the original volume of culture. The washed cells, resuspended in the appropriate medium, were used as the experimental culture.

Induction of sucrose phosphorylase. A portion of cell suspension (sufficient to yield a final concentration of 0.4 ± 0.04 mg of bacterial protein per ml) was added to either complete medium or phosphate buffer containing 0.2% sucrose.

The enzyme activity was assayed by the method of Doudoroff (3), by use of enzyme extracts prepared by sonic treatment in an ultrasonic disintegrator (model 3100; Measuring & Scienfic Equipment, Ltd., London, England). Cell suspensions were subjected to 20 kc/min for 8 min at an output of 500 w. Enzyme extracts containing more than 10 units of enzyme per ml were incubated at 30 C for 20 min; less-active enzyme extracts were incubated for 1 hr. Protein was determined by the method of Lowry et al. (7).

Determination of the incorporation of ^{14}C amino acid into proteins. The cells in 20 ml of culture were precipitated by the addition of 10 ml of 15% trichloroacetic acid, and then the cells were washed with 30 ml of cold 5% trichloroacetic acid. (This simplified procedure was found to yield the same ^{14}C activity in the final product as did the customary lengthier process involving hot trichloroacetic acid, alcohol, and ether.) The precipitate was dissolved in 0.5 ml of 88% formic acid, and an 0.4-ml sample of the formic acid mixture was added to 10 ml of scintillation fluid;

the ^{14}C activity was determined in a Tri-Carb (model 3324) spectrometer (16). Under these conditions, the counting efficiency in the formic acid mixture was found to be 50%.

RESULTS

Decrease in net protein synthesis upon addition of exogenous valine. Lactate-grown cells were subcultured on a complete medium containing various amounts of DL-valine, with sucrose as the main carbon source. The protein content of each culture was determined immediately after inoculation and again after 2 hr, the difference in protein content representing the net protein synthesis during that period. In most experiments, cultures grown without exogenous valine increased their total protein by about 40 to 50% in 2 hr. As the concentration of valine increased, however, net protein synthesis decreased; at a concentration of 0.3 μmole of valine per mg of initial protein, the increment was only about one-third that of the control (Fig. 1). Higher valine concentrations did not further reduce protein synthesis. At high concentrations, the inhibitory effect of valine is independent of cell concentration; this is not the case at lower valine concentrations. Therefore, the results here are expressed in terms of initial bacterial protein. Frequently, as in the experiment shown here, low concentrations of valine (below about 0.15 μmole/mg of initial protein) actually increased net protein synthesis slightly.

Both D- and L-valine were inhibitory, but L-valine was effective at lower concentrations than D-valine. This suggests that L-valine is responsible for the inhibition and that D-valine is probably converted to the L isomer by a racemase, although

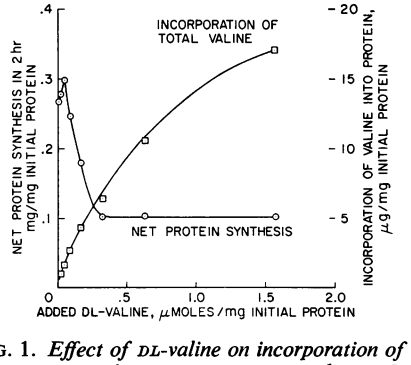

FIG. 1. *Effect of DL-valine on incorporation of valine into proteins and on net protein synthesis. Samples (1 ml) of Pseudomonas saccharophila were added to flasks containing 25 ml of complete medium, 0.2% sucrose, 0.02 μc of L-valine-1-^{14}C/ml, and various amounts of DL-valine. The initial bacterial protein concentration was 0.44 mg/ml. Incubation was for 2 hr.*

no valine racemase was found in *P. aeruginosa* (12). It is also possible that the site at which L-valine binds lacks specificity and has a lower affinity for D-valine.

Sixteen amino acids were tested under the same conditions, and only D- and L-valine or L-isoleucine inhibited net protein synthesis. However, when valine and isoleucine were added together in a ratio of 3:1, no inhibition was observed. These results suggest that valine acts by feedback inhibition as proposed by Umbarger and Brown (15).

Effect of valine on the incorporation of ^{14}C-valine into proteins. To analyze the mechanism of valine inhibition, the effect of valine on the incorporation of ^{14}C-labeled amino acids was studied. To the culture described above (Fig. 1), L-valine-1-^{14}C was added to give a final activity of 0.02 μc/ml. At the end of 2 hr, samples were removed, to determine total protein and radioactivity. Figure 1 shows that valine incorporation into protein is a function of the concentration of added valine, and that this incorporation is not directly related to the inhibition of net protein synthesis. Over the range of valine concentrations causing severe inhibition, valine is nevertheless incorporated into proteins in large amounts.

In this experiment, when exogenous valine was present at 1.0 μmole/mg of initial protein, the valine actually incorporated into total proteins was 14.2% of the observed net protein increase, and at 1.5 μmoles it approached 17%. Amino acid analyses on normal as well as valine-inhibited cells showed that both types of cells contained 7.5% valine in their total protein. Thus, the "excess" incorporation of ^{14}C-valine could be used to calculate protein turnover. Table 1 shows the progressive increase in "turnover" rates as the valine concentrations increased. At 1.56 μmoles of valine per mg of initial protein, this rate approaches the turnover rate of the resting cells.

Effect of valine on turnover of ^{14}C-leucine in protein. The effect of valine on protein turnover was also examined by studying the effect of added valine on the incorporation of ^{14}C-leucine into proteins, and on the release of leucine from prelabeled cells. In the first experiment, bacterial cells were handled exactly as described in the previous section, except that the ^{14}C-valine was replaced by an equal amount of L-leucine-1-^{14}C, and L-valine was used as the inhibitor of net protein synthesis. Table 2 demonstrates again the inhibitory effect of added valine. Furthermore, incorporation of L-leucine actually increases substantially under these circumstances. This suggests that, as the valine concentration is increased, leucine is incorporated into "turnover protein."

The effect of L-valine on the release of radioactivity from cells prelabeled with leucine-*1*-^{14}C was also examined. Cells labeled with ^{14}C-leucine were harvested and subcultured in sucrose growth medium in the presence of a large quantity of unlabeled leucine and various amounts of L-valine. Samples from each culture were precipitated with trichloroacetic acid at a final concentration of 5%, and the radioactivity of the acid-soluble fractions was determined. A progressive release of radioactivity was observed during the growth up to 2 hr (Fig. 2). Subsequently, the radioactivity began to decrease, probably because of reincorporation of the labeled amino acid into protein. Figure 2 shows that more ^{14}C activity was released to the acid-extracted fractions as the valine concentration increased. The turnover rates for cellular protein calculated from these data agree reasonably well with those obtained from studies on the incorporation of ^{14}C-valine. For example, at 1.53 μmoles of valine, the turnover calculated by the release of radioactivity, during the first 30 min, is 6.7% per hr; at 1.01 μmoles of valine, the rate is 4.5%.

TABLE 1. *Incorporation of valine into proteins of Pseudomonas saccharophila*[a]

DL-Valine concn (μmoles/mg of initial protein)	Calculated turnover protein[b] (μg/mg of initial protein)	Calculated turnover rate (% per hr)
Growing cells		
0.05	—	0
0.36	0	0
0.40	8	0.4
0.50	20	1.0
1.00	90	4.5
1.56	126	6.3
Nongrowing cells[c]		
0.05	112	5.6
1.80	170	8.5

[a] Data on growing cells were obtained from the experiment shown in Fig. 1.

[b] Turnover protein was calculated by comparison of the incorporation of ^{14}C-valine excess to that incorporated into net proteins synthesized, with the valine concentration at 7.5% of protein (Young and Klein, *unpublished data*).

[c] Result from a parallel culture aerated in 0.2% sucrose in 0.033 M phosphate buffer. These results are comparable to those reported for this organism in the absence of a carbon source (6).

TABLE 2. *Effect of L-valine on incorporation of ^{14}C-leucine into protein*[a]

L-Valine concn (μmoles/mg of initial protein)	Net protein synthesis (μg/mg of initial protein)	L-Leucine incorporation[b]
0	105	1.39
0.022	84	1.58
0.044	94	1.51
0.088	89	1.75
0.176	70	2.17
0.264	64	2.35
0.352	45	3.54
0.440	30	5.18

[a] Samples (1 ml) of *P. saccharophila* in 0.033 M phosphate buffer were added to flasks containing 25 ml of complete medium, with 0.2% sucrose, 0.02 μc of L-leucine-*1*-^{14}C/ml, and various amounts of L-valine and incubated for 2 hr. The initial bacterial protein concentration was 0.44 mg/ml of culture.

[b] Expressed as counts × mg of new net protein^{-1} × min^{-1} × 10^{-5}.

Effect of L-valine on the formation of inducible sucrose phosphorylase. Since valine strongly inhibited net protein synthesis, it appeared of interest to examine whether valine similarly inhibited the induced synthesis of a specific protein, particularly an enzyme necessary to generate energy from sucrose. Table 3 shows that L-valine indeed inhibited the formation of sucrose phosphorylase. The amount of this enzyme synthesized is directly proportional to net protein synthesis and is not related to the total protein synthesis, which includes proteins synthesized by turnover. This suggests that turnover of proteins is not related to the formation of sucrose phosphorylase. "Turnover protein" here may thus

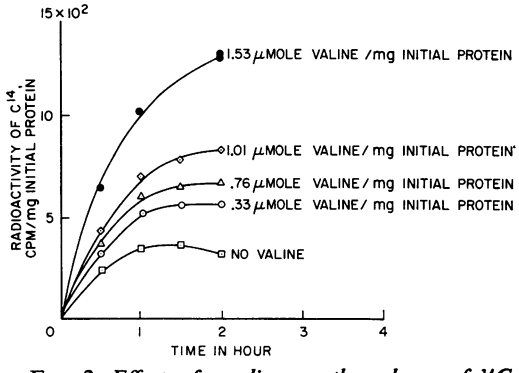

FIG. 2. *Effect of L-valine on the release of ^{14}C-leucine from prelabeled cells. Bacterial cells were prelabeled with leucine-1-^{14}C in growth medium (8 μc of ^{14}C-leucine per 100 ml of medium) for 18 hr and then resuspended in growth medium containing various amounts of valine. After 2 hr of incubation, the radioactivity in the trichloroacetic acid-soluble material was analyzed. The initial bacterial protein concentration of the culture was 0.46 mg/ml.*

TABLE 3. *Effect of valine on induction of sucrose phosphorylase*[a]

Concn of L-valine (μmoles/mg of initial protein)	Net protein synthesis (mg/mg of initial protein)	Sucrose phosphorylase (units/mg of protein)	Sucrose phosphorylase-net protein synthesis ratio
0	0.758	1.93	2.55
0.058	0.752	1.86	2.47
0.117	0.704	1.56	2.22
0.588	0.474	1.18	2.48
1.176	0.460	1.01	2.20
2.352	0.375	0.88	2.35

[a] In this experiment, 5 ml of *P. saccharophila* in 0.033 M phosphate buffer was added to 45 ml of complete medium containing 0.2% sucrose and various amounts of L-valine. Sucrose phosphorylase of cultures was assayed after 4 hr of incubation. The initial bacterial concentration was 0.40 mg/ml of culture.

represent a labile class of protein, recycling nonspecifically, as suggested by Pine (13).

DISCUSSION

In *P. saccharophila*, as opposed to *E. coli* K-12, inhibition by L-valine is mild and transient except under rather specific conditions. The experiments reported here were conducted on cells transferred from a lactate to a sucrose medium. Under these conditions, the organisms must form the enzyme, sucrose phosphorylase, before they can utilize the new carbon source. Thus, the cells are deprived of a carbon source at the same time they are (presumably) inhibited in the accumulation of at least one amino acid. The lack of a usable carbon source seems to be the more important factor, since addition of valine to cells subcultured from a sucrose medium (and, therefore, not required to form inducible enzymes to obtain energy) inhibits net protein synthesis for only a brief period at high concentrations. By contrast, when induction is a prerequisite for growth, valine inhibition lasts for several hours, after which the cells gradually recover.

In the experiments reported here, L-valine or L-isoleucine inhibits net protein synthesis. However, a mixture of valine and isoleucine in a ratio of 3:1 abolishes the inhibitory effects. It is interesting to note that this ratio is the same as that found in the free amino acid pool (Young and Klein, *unpublished data*) of this organism and suggests that a critical ratio of these amino acids is required for optimal protein synthesis.

The incorporation of ^{14}C amino acids and the release of radioactivity from prelabeled cells show that valine also stimulates protein turnover. It is not evident how the restriction of a carbon source could precipitate the process of protein turnover. Deprivation of carbon, nitrogen, or specific amino acids can initiate protein degradation (8, 10). It has been suggested that proteolytic enzymes responsible for the degradative phase of protein turnover may be inactive in growing cells because of binding to particles (lysosomes?), and that this binding requires the expenditure of energy (9). If the flow of energy is stopped, the proteolytic enzyme would thus be released and protein breakdown initiated. It is also possible that a specific amino acid(s) or peptide is present in the soluble pool of growing cells and that this substance prevents proteolysis. Then, under conditions that restrict the synthesis of this inhibitor (i.e., carbon starvation), the proteolytic enzyme is free to act on available substrates.

LITERATURE CITED

1. ARMSTRONG, F. B., AND R. P. WAGNER. 1963. Repression of the valine-isoleucine pathway in Salmonella. Proc. Natl. Acad. Sci. U.S. **49**:628–633.
2. BONNER, D. 1946. Further studies on mutant strains of neurospora requiring isoleucine and valine. J. Biol. Chem. **166**:545–554.
3. DOUDOROFF, M. 1955. Disaccharide phosphorylases, p. 225–231. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
4. FREUNDLICH, M., R. O. BURNS, AND H. E. UMBARGER. 1962. Control of isoleucine, valine, and leucine biosynthesis. I. Multi-valent repression. Proc. Natl. Acad. Sci. U.S. **48**:1804–1808.
5. GLADSTONE, G. P. 1939. Interrelationships between amino acids in nutrition of *B. anthracis*. Brit. J. Exptl. Pathol. **20**:189–200.
6. KLEIN, H. P. 1963. Synthesis of enzymes in resting cells. Ann. N.Y. Acad. Sci. **102**:637–654.
7. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**: 265–275.
8. MANDELSTAM, J. 1958. Turnover of protein in growing and nongrowing populations of *Escherichia coli*. Biochem. J. **69**:110–119.
9. MANDELSTAM, J. 1960. The intracellular turnover of protein and nucleic acids and its role in biochemical differentiation. Bacteriol. Rev. **24**: 289–308.
10. MANDELSTAM, J., AND H. HALVORSON. 1960. Turnover of protein and nucleic acid in soluble and ribosome fractions of nongrowing *Escherichia coli*. Biochim. Biophys. Acta **40**:43–49.
11. MARKOVITZ, A., AND H. P. KLEIN. 1955. Some aspects of the induced biosynthesis of alpha-

amylase of *Pseudomonas saccharophila*. J. Bacteriol. **70**:641–648.
12. NORTON, J. E., AND J. R. SOKATCH. 1966. Oxidation of D- and L-valine by enzymes of *Pseudomonas aeruginosa*. J. Bacteriol. **92**:116–120.
13. PINE, M. J. 1965. Heterogeneity of protein turnover in *Escherichia coli*. Biochim. Biophys. Acta **104**:439–456.
14. TEMPLE, R. J., H. E. UMBARGER, AND B. MAGASANIK. 1965. The effect of L-valine on enzyme synthesis in *Escherichia coli* K-12. J. Biol. Chem. **240**:1219–1224.
15. UMBARGER, H. E., AND B. BROWN. 1955. Isoleucine and valine metabolism in Escherichia coli. V. Antagonism between isoleucine and valine. J. Bacteriol. **70**:241–248.
16. YOUNG, H. L., AND I. S. EDELMAN. 1962. Carbohydrate metabolism and electrolyte content of rabbit diaphragm in vitro: effect of Na pyruvate. Am. J. Physiol. **203**:1008–1014.